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Electron Paramagnetic Resonance of Hemoglobins Gamma-Irradiated at 77K

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The effects of high energy electron and gamma irradiation on hemoglobin have been studied at dry state by electron paramagnetic resonance spectroscopy at a temperature range from 77 K to room temperature. In the irradiated samples of oxyhemoglobin, methemoglobin, globin, hemin, and a mechanical mixture of globin with hemin, the radicals are found to be localized in the globin moieties. No indication of radiation damages being transfered from the protein to the heme part, and vice versa, is given. The spectra of hemoglobins at 77 K are attributed to a sum of the EPR spectra of various radicals of two types distributed randomly in the constituent amino acid residues: –ĊHR radicals produced by scission of polypeptide main chains and –NH–CH(Ṙ)–CO– radicals in side chains R. By warming, the former radicals are converted to the type –NH–ĊR–CO–, predominantly to –NH–ĊH–CO– of the glycine residue, while the latter are transferred or disappeared. The selective radical formation on the glycine residues at room temperature are attributed to the result of a series of radical transfer reactions initiated by radicals produced by main chain scission randomly in the protein molecule.

INTRODUCTION

When proteins are exposed to ionizing radiation in their solid state, two types of radicals which are stable at room temperature are usually observed by EPR (electron paramagnetic resonance). They are an –NH–CH(Ṙ)–CO– radiacal formed in the side chain R of an amino acid residue (Group I), and an –NH—ĊR–CO– radical with an unpaired electron mainly localized on the α-carbon of polypeptide main chains (Group II), both being formed by liberation of a hydrogen atom. The most dominant free radicals of Group I are –NH–CH(CH_2–Ṡ)–CO– radicals produced in cysteine or cystine residues, while those of Group II are the –NH–ĊH–CO– radicals produced in glycine residues.[1] This selectivity in radiation effects on proteins appears to originate during the primary steps in the processes.

From the EPR studies on cystine dihydrochloride, it has been found that the primary radicals are selectively localized on the S–S bond even at 77 K and that they change into –Ṡ radicals of Group I.[2,3]

On the other hand, in the previous works on glycylglycine[4] and several polyamino acids and dipeptides,[5] it has been presented that the primary radicals are produced by main chain scissions and that they change into stable radicals of Group II at room temperature. The mechanism has also been proposed that a bond between an α-carbon and a nitrogen atom in the main chain is temporarily broken to produce a ĊHR–CO– radical on

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irradiation, and the radical abstracts an α-proton from undamaged chains at higher temperature, thus producing an –NH–ĊR–CO– radical.

In this paper we investigate the structure of the unstable free radicals of irradiated hemoglobin and discuss the above mechanism in conjunction with the selectivity of radiation effects on proteins in terms of EPR results.

MATERIALS AND METHODS

Preparations of oxyhemoglobin from bovine blood were carried out by the method of Heidelberger[6] with the modification described by Kikuchi.[7] The final dense precipitates were lyophilized, a part of which was dissolved in distilled water and identified as the oxy-form by its optical absorption spectrum in visible light regions. Methemoglobin identified optically, globin, and hemin were purchased from Nakarai Chemicals, Co. Ltd., Kyoto.

Powdered samples were sealed at a pressure lower than 10^{-3} mmHg in glass sample tubes of about 4 mm inner diameter, and irradiated in liquid nitrogen either with an electron beam from a Van de Graaff Accelerator at a dose rate of 10^5 rads per second to a total dose of 1×10^7 rads, or with γ-rays from a Co-60 source at a dose rate of 6×10^4 rads per hour to a total dose of 1×10^6 rad.

EPR spectra were obtained with a Varian V 4560 EPR spectrometer or with a JEOL 3 BX X-band ESR spectrometer, with 100 Kc/sec modulation. The first derivative of the absorption signal was recorded, and for quantitative studies some of the spectra were numerically integrated. Standard samples of DPPH (diphenylpicrylhydrazyl), and Mn^{++} ion were used for calibration purpose. Temperature-dependent changes of the spectra were studied by the two methods. One method employed a Varian variable temperature accessary to observe spectra at various temperatures. The other method was to observe spectra always at 77 K after successive stepwise heat treatments at dry-ice and room temperatures.

RESULTS

Oxyhemoglobin

EPR spectra of oxyhemoglobin irradiated at 77 K and after heat treatments are shown in Fig. 1. At 77 K and after annealing at dry-ice temperature they give similar spectra with odd hyperfine lines centered at $g=2.002$, whereas after warming at room temperature a doublet spectrum, with a splitting of 17 gauss and centered at $g=2.004$, is observed. The radical concentration remaining after heat treatments is estimated to be 100% after one minute, and 57% after 150 minutes of annealing at dry-ice temperature; then 50% after one minute and 20% after 10 minutes at room temperature, respectively.

The odd-line spectra at 77 K and at dry-ice temperature are attributable to the sum of the spectra of various radicals, as is discussed below.

When the sample was warmed for one minute at room temperature after 150 minutes' annealing at dry-ice temperature, the radical concentration decreased slightly, about 7% (57 to 50%), but the spectrum changed remarkably from that with odd-line to even-line components. The spectrum of the latter was subtracted from the former, and the resultant

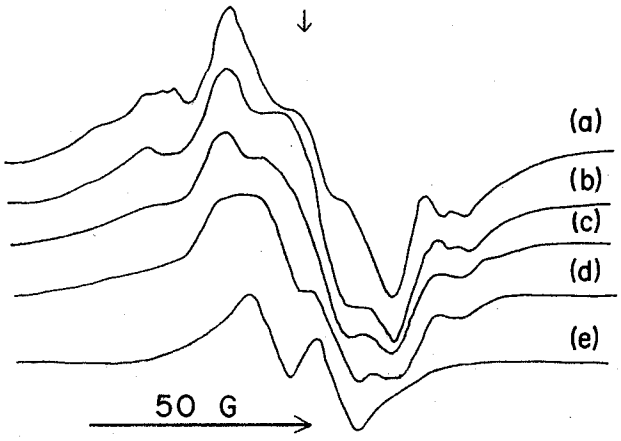

Fig. 1. EPR spectrum of oxyhemoglobin irradiated and observed at 77 K after successive heat treatments. (a) before heat treatment, (b) after heat treatment for one minute, (c) for 150 minutes at dry ice temperature, for one minute (d) and 60 minutese (e) at room temperature. The arrow at the top indicates the resonance position of the DPPH signal (g=2.0036).

difference spectrum showed a 1 : 4 : 6 : 4 : 1 quintet with about 20 G hf splitting width. The species that disappears is attributable to the radical $-NHCH(CH_2CH_2CH_2\dot{C}H_2)CO-$ formed in the side chain of lysine residues, because this radical shows a 1 : 4 : 6 : 4 : 1 quintet with about 20 G splitting at 77 K, and also disappears very rapidly at room temperature, as found by Drew and Gordy in their study on irradiated polylysine.[8] It is interesting to note that 48 lysines in 582 residues of bovine hemoglobin residues (8%) as seen in Table I[9,10], is almost equal to the decreased radicals (7%). This seems to suggest

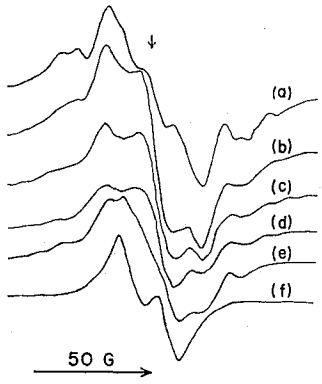

Fig. 2.

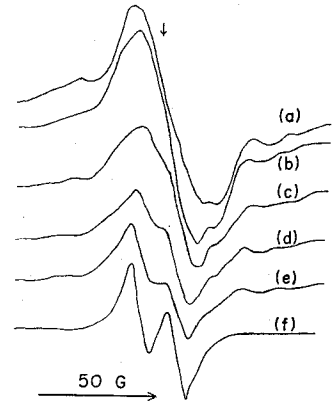

Fig. 3.

Fig. 2. EPR spectrum of methemoglobin irradiated at low temperature and observed at (a) −196°C, (b) −150°C, (c) −100°C, (d) −55°C, (e) −15°C, and (f) 25°C. The arrow same as above.

Fig. 3. EPR spectrum of the mechanical mixture of globin with hemin irradiated at 77 K and observed at, (a) −196°C, (b) −150°C, (c) −100°C, (d) −55°C, (e) −15°C, and (f) 25°C. The arrow same as above.

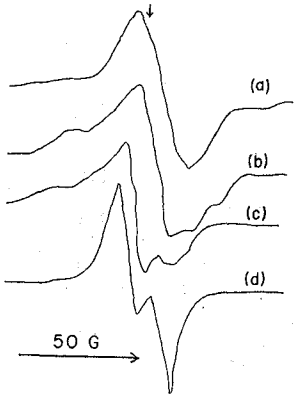

Fig. 4.

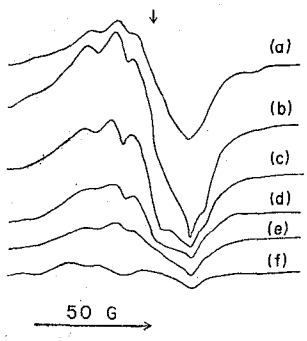

Fig. 5.

Fig. 4. EPR spectrum of globin irradiated at 77 K and observed at, (a) −196°C, (b) −150°C, (c) −15°C, and (d) 25°C. The arrow same as above.

Fig. 5. EPR spectrum of hemin irradiated at 77 K and observed at, (a) −196°C, (b) −150°C, (c) −100°C, (d) −55°C, (e) −15°C, and (f) 25°C. The arrow same as above. The spectra are measured with 20 times as large amplitude as those used for the above four samples.

that radicals initially produced in hemoglobin are ramdomly distributed in the molecule in proportion to the amino acid composition.

The last doublet that survived after warming at room temperature is identified as that from the radical of structure –NH–ĊH–CO–, since this doublet has the same g-value of 2.004, hf splitting of 17 G, and stability at room temperature as the doublet obtained with polyglycine radicals.[5,8]

Methemoglobin

Methemoglobin was irradiated at 77 K, and its EPR spectra were observed at various temperatures by using the temperature varying accessary. As given in Fig. 2, they show similar changes to the irradiated oxyhemoglobin, in the shape of hf structures, g-values, splitting widths, and the stability, suggesting that the difference in the heme part of methemoglobin from oxy-form apparently has no relation to the radical produced.

Mechanical mixtures of globin and hemin

A mechanical mixture of globin and the equivalent hemin γ-irradiated changes spectra depending on temperatures in the same way as hemoglobin as seen in Fig. 3.

Globin

The spectra of globin, the protein moiety of hemoglobin, γ-irradiated resemble closely that of the hemoglobins and the mixture of globin with hemin as shown in Fig. 4. This suggests that paramagnetic centers are located predominantly in the protein part in the case of above three samples.

Hemin

Figure 5 shows the spectra of γ-irradiated hemin at various temperatures. They have no similarity to those of the above four samples. The radical yield is below a twentieth of those of hemoglobin.

DISCUSSION

The spectra of oxyhemoglobin, methemoglobin, mechanical mixtures of globin with hemin, and globin, at 77 K show essentially the same broad symmetric lines with odd hf components. These results are somewhat unique compared with those observed in the case of ribonuclease, lysozyme, and trypsin which were broad singlet lines.[11,12] Whereas the EPR centers giving rise to these singlet at low temperature were not identified but assumed to be the precursors for the secondary radicals,[12] here in the case of hemoglobins it seems to be easier to assign the structures of initially formed radicals because of their comparatively well resolved hf lines.

It seems very probable that initially produced radicals distribute randomly in the irradiated protein molecule, as suggested above by the disappearance of lysine-type radicals proportional to the lysine content. The random distribution of radicals in irradiated proteins has also been postulated by Sugimoto *et al.* based on the study of a γ-irradiated single crystal of myoglobin which showed only slight anisotropy in the hf coupling constant and the g-value in the EPR spectra.[13] The spectra at low temperature can then be the result of the superposition of independently observed spectra in irradiated copolymers of constituent amino acids at low temperature.

It has been reported by Drew and Gordy[8] that most of fifteen polyamino acids they studied exhibit odd hf lines at 77 K. These spectra were attributed to arise from charged or ionized molecules, their structures being unidentified. As to the major component residues of bovine hemoglobin shown in Table I, polyleucine (12.7% of residues) shows a triplet-like complex pattern, polyalanine (12.4%) a quintet, polyvaline (10.3%) a triplet, polyaspartic acid (9.3%) a broad triplet, polylysine (8.3%) a quintet, and polyglycine (6.9%) a broad triplet. In our previous work on irradiated polyglycine, polyalanine, and polyglutamic acid, the initially formed radicals were assigned to be ĊHR–CO– produced by N-C bond scission in polypeptide backbones, which show odd hyperfine peaks centered at $g=2.002$.[5]

The sum of these spectra will result in a broad complex spectrum with odd hyperfine

Table I. Amino Acid Composition of Bovin Hemoglobin Expressed as Moles of the Residue Per One Mole of Hemoglobin[a]

Leucine	74	12.7%	Histidine	32	5.5%
Alanine	72	12.4	Threonine	28	4.8
Valine	60	10.3	Proline	20	3.4
Aspartic acid	54	9.3	Arginine	14	2.4
Lysine	48	8.3	Isoleucine	10	1.7
Glycine	40	6.9	Tyrosine	10	1.7
Serine	36	6.2	Methionine	8	1.4
Glutamic acid	34	5.8	Tryptophan	6	1.0
Phenylalanine	34	5.8	Cysteine	2	0.3
			Total	582	99.9%

a. Schroeder *et al.* (see ref. 9 and 10).

components centered near $g=2.002$, which is what has been observed in irradiated hemoglobins at low temperature.

The spectral changes by the heat treatments can also be explained by the scheme presented from the results on polyamino acids.[5] Radicals formed in polyamino acids at 77 K were converted by heating to the type of $-NH-\dot{C}R-CO$, which showed even hyperfine lines and g-values near 2.004. The spectra of irradiated hemoglobins changed similarly from odd to even hf lines and g-values from 2.002 to 2.004 after the heat treatments.

Radical concentration remaining at room temperature was, however, 20% of that which existed at 77 K. The reduction of 80% may be due to the disappearance of the radicals due to recombination in the heating process. During radical conveirsion in polyamino acids, about half of the radicals disappeared.[5] Radicals formed in the side chains of amino acid residues might be labile and disappear at room temperature, as do radicals in lysine residues.[8]

There is a possibility that some radicals transfer to the most stable one in irradiated hemoglobins at higher temperatures. The concentration of the radical at room temperature, which was attributed to the glycine radical, was 20% of the initial concentrations, which is about three times as much as the glycine content in bovine hemoglobin (7%). Considering the primary random formation of radicals in the irradiated protein in proportion to the amino acid composition, as discussed above, this seems to suggest that some of the unstable radicals transfer to glycine residues during the heat treatment from 77 K to room temperature through such a process previously proposed for the radical conversion in irradiated glycylglycine and polyamino acids.[4,5] The methylene group hydrogens of glycine residues must be more reactive than any other residues that have a bulky side chain, and glycine residues in proteins may behave like traps for radiation damages, just like cysteine residues. However, it must be emphasized that the selective formation of stable radicals on glycine in irradiated proteins is the result of series of intermolecular radical transfer reactions initiated by the unstable radicals produced by main-chain scission, but not the primary localization of damages at glycine residues, in contrast to the case of radicals on cysteines.

Thus, in a protein irradiated at room temperature, particular stable radical species are observed, which are glycine radicals in the case of hemoglobins, or which are cystein radicals in those proteins which contain cysteine in large ammount.[1]

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